

## ORIGINAL ARTICLE

# Colonization of peanut roots by biofilm-forming *Paenibacillus polymyxa* initiates biocontrol against crown rot disease

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**Abstract****Aim:** To investigate the role of biofilm-forming *Paenibacillus polymyxa* strains in controlling crown root rot disease.**Methods and Results:** Two plant growth-promoting *P. polymyxa* strains were isolated from the peanut rhizosphere, from *Aspergillus niger*-suppressive soils. The strains were tested, under greenhouse and field conditions for inhibition of the crown root rot pathogen of the peanut, as well as for biofilm formation in the peanut rhizosphere. The strains' colonization and biofilm formation were further studied on roots of the model plant *Arabidopsis thaliana* and with solid surface assays. Their crown root rot inhibition performance was studied in field and pot experiments. The strains' ability to form biofilms in gnotobiotic and soil systems was studied employing scanning electron microscope.**Conclusion:** Both strains were able to suppress the pathogen but the superior biofilm former offers significantly better protection against crown rot.**Significance and Impact of the Study:** The study highlights the importance of efficient rhizosphere colonization and biofilm formation in biocontrol.**Introduction**

Crown rot disease of peanut (*Arachis hypogaea* L.) caused by *Aspergillus niger* is the most important disease in Egypt and several other temperate countries (Haggag and AboSedera 2000; Elwakil 2003; Carina *et al.* 2006). Peanut seeds infected with seed-borne fungi have been reported to produce seed abortion, shrunken seeds, reduced seed size, seed rot, seed necrosis, seed discoloration, reduction of germination capacity and physiological alteration of seed (Elwakil 2003). In-furrow application of pentachloronitrobenzene (PCNB) and azoxystrobin at planting have been used to fight the disease. Owing to environmental concerns there is considerable interest in finding alternatives to the chemical pesticides. Biological control represents an attractive alternative because of the many concerns about pesticide use in general.

There are many reports that certain rhizobacteria can promote plant growth and reduce detrimental effects of various stresses under controlled conditions (Lucy *et al.*

2004). Yet one has to admit that in agro-ecological systems successful biotechnological applications of natural microbial isolates are rare. This is due to the complexity of natural microbial systems and also to the lack of a comprehensive understanding of them (Emmert and Handelsman 1999; Gau *et al.* 2002; Gamalero *et al.* 2003).

During our former activities we have studied isolates of the plant-beneficial bacterium *Paenibacillus polymyxa* from the rhizosphere of spring wheat (cultivar Draband) and barley (cultivar Ingrid) (Lindberg and Granhall 1984). *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*; Ash *et al.*, 1994), a common soil bacterium, belongs to the group of plant growth-promoting rhizobacteria (Timmusk and Wagner 1999; Timmusk *et al.* 1999, 2003, 2005; Timmusk 2003; Alvarez *et al.* 2006; He *et al.* 2007). Frequently, bacteria live in the environment as biofilms, which are highly structured, surface-attached communities of cells encased within a self-produced extracellular polymeric substance matrix (O'Toole *et al.* 1999, 2000; Branda *et al.* 2005; Kolter and Greenberg 2006). Using

scanning electron microscopy (SEM) and epifluorescent microscopy we characterized the bacterial biofilm formation around the roots at various time intervals using a model system (Timmusk and Wagner 1999) and suggested that this could be exploited to protect plants from root pathogens (Timmusk and Wagner 1999; Timmusk *et al.* 2005). Bacterial biofilms established on plant roots could protect the colonization sites and act as a sink for the nutrients in the rhizosphere, hence reducing the availability of root exudate nutritional elements for pathogen stimulation or subsequent colonization on the root (Weller and Thomashow 1994). In this study, we report the isolation of bacterial strains for the biocontrol of crown root rot disease. Two *P. polymyxa* isolates were characterized and tested for peanut plant colonization and for their ability to antagonize *A. niger* in field and greenhouse experiments and on agar plates. In addition, the strain colonization and biofilm formation ability were assayed in an *Arabidopsis thaliana* gnotobiotic system (Timmusk and Wagner 1999) and on abiotic solid surfaces (Friedman and Kolter 2004).

## Materials and methods

### Isolation and identification of *P. polymyxa* and *A. niger*

The *A. niger*-suppressive soil at Noubaria was screened for bacteria that produce antimicrobial agents. Briefly, three peanut plants from different locations were harvested and their roots were shaken to remove loosely attached soil. The peanut rhizosphere root samples together with attached soil were suspended in 0.1% sterile peptone water and homogenized using the FastPrep Instrument (BIO 101; Bio 101 Systems, Carlsbad, CA). The suspensions were pasteurized (10 min at 80°C), serially diluted and passed through a hydrophobic grid membrane filter with a pore size of 0.45 µm (ISO-GRIDNeogen, Corporation, Lansing, Lexington, MI, USA). Bacteria and fungi on the membranes were grown into colonies by placing the membranes onto tryptose agar and potato dextrose agar (PDA) and incubating for 48 h at 30°C. The colony-carrying membranes were then removed. The incubated agar plates were overlaid with a soft agar medium seeded with *Escherichia coli* K12. The medium consisted of nutrient broth supplemented with 0.6% yeast extract and 0.75% agar. The overlaid plates were incubated at 37°C for an additional 48 h to show inhibition areas. Isolates corresponding to inhibition areas were located on the membrane filter and streaked onto nutrient agar (NA) plates.

Tests for the biochemical properties of the antagonistic bacteria were performed. The analyses including catalase, oxidase and urease reactions, nitrate reduction, gelatine lignification, starch hydrolysis, glucose fermentation,

indole production and H<sub>2</sub>S formation and the carbohydrate fermentation pattern of the isolates was determined using a biochemical test kit (API 50CH strips and API CHB/E medium; BioMerieux, Inc., Durham, NC, USA). The results were checked after incubating strains at 30°C for 24 and 48 h, and the bacteria were identified by referring to the database provided by the kit manufacturer.

### Genomic identification of *P. polymyxa* was performed by 16S rRNA gene sequencing

A universal primer set specific for bacterial 16S rRNA fD1 and rD1 (Weisburg *et al.* 1991) was used to amplify the 16S rRNA gene. The PCR amplification was performed using a *Taq* DNA polymerase kit (Qiagen, Valencia, CA, USA) under the following conditions: after an initial 3-min incubation at 95°C the mixture was subjected to 30 cycles, each of 1 min at 95°C, 30 s at 52°C and 2 min at 72°C. A final extension was performed at 72°C for 10 min. The amplified 16S rRNA was purified using a DNA extraction kit (QIAquick Qiagen). Subsequently, it was ligated into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and transformed into *E. coli* DH5a cells via electroporation. The recombinant plasmid was harvested from an overnight LB culture using QIAprep Spin Miniprep kit columns (Qiagen) and sequenced (3730xl DNA Analyser, Applied Biosystems, Foster City, CA, USA) using T7 and SP6 promoter sequences as primers. The derived 16S rRNA gene sequence (approx. 1.5 kb) was compared to known bacterial sequences in NCBI GenBank using BLAST.

*Aspergillus niger* was isolated from peanut plants (Giza 4 cv.) naturally infected with crown root disease in a field in Noubaria, Bohera Governorate. Infected roots and seeds were collected, surface-sterilized with 0.5% sodium hypochlorite solution for 1–2 min and placed on PDA medium (Difco) at 24°C. After 2 days of growth the colonies were transferred to fresh agar plates and grown for 4 days for morphological identification by microscopy. The genomic identification was performed by PCR as described above employing the primer set and conditions designed for conserved regions of *A. niger* internal transcribed spacer (521 bp; Sugita *et al.* 2004).

### Preparation of *P. polymyxa* and *A. niger* inoculants

*Paenibacillus polymyxa* B5 and B6 were grown in nutrient broth at 30°C for 24 h. Cells were harvested by centrifugation (9000 g, 10 min), washed twice with sterile water, and then resuspended in phosphate-buffered saline (PBS) to a density of 10<sup>5</sup> bacteria ml<sup>-1</sup>.

*Aspergillus niger* mycelial agar plugs were transferred to PDA plates and incubated at 24°C for 72 h to prepare

conidia spores. The spores were harvested by washing with 5 ml sterile water, containing 0.01% Tween 80, filtered through a 30  $\mu\text{m}$  nylon filter and centrifuged for 5 min at 2600 rpm. Pellets were dissolved in PBS to a density of  $10^4$  spores  $\text{ml}^{-1}$ .

### Field experiments

Two successive field experiments were carried out during the 2005 and 2006 seasons to evaluate the *P. polymyxa* isolate potential to protect peanut plants against infection with *A. niger*. The field station where the indigenous *P. polymyxa* population was absent was chosen for experiments. Trials were conducted at Experimental Farm of the National Research Centre at El Kanater, Kalubaiya Governorate under natural infection conditions. A split-plot design with four replicates per treatment was used. Each individual plot consisted of a five-row bed. Peanut seeds (Giza 4 cv.) were sown with 30 cm distance between plants and 50 cm between rows. The seeds were soaked for 30 min in cell suspensions of *P. polymyxa* at a density of  $10^5$  cells  $\text{ml}^{-1}$ . One hundred seeds were used for each replicate and commonly recommended methods of planting and watering were followed. The crown rot incidence was estimated over 140 days during different growth periods. Nodule dry weight, plant dry weight and yield of peanut plants were estimated.

*Aspergillus niger* and *P. polymyxa* presence and population size in the peanut rhizosphere were checked before the planting and then monthly during 140 days after planting. *Paenibacillus polymyxa* populations were estimated as colony counts from the rhizosphere macerates on starch/basic fuchsin medium (Yuen *et al.* 1991). Five peanut plants from different locations were harvested and their roots were shaken to remove the loosely attached soil. Plants were placed in a new plastic bag, transferred to the laboratory and stored at  $+4^\circ\text{C}$  until processing the next day. The peanut rhizosphere root samples together with attached soil were suspended in 0.1% sterile peptone water and homogenized using the FastPrep Instrument (BIO 101). Macerates were dilution plated on the medium which consisted of 1.0 g  $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.2 g KCl, 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 g potato starch, 1.5 g yeast extract and 18 g agar  $\text{l}^{-1}$ . After autoclaving, aqueous solutions of basic fuchsin,  $\text{FeCl}_3$  and cycloheximide were added to the medium to final concentrations of 2, 10 and 100 mg  $\text{l}^{-1}$ , respectively. *Paenibacillus polymyxa* was differentiated from other bacteria based on the large, raised pink colonies, surrounded by zones of starch hydrolysis, which developed after 4 days at  $25^\circ\text{C}$ . Further verification was performed selectively by microscopy or real-time PCR (S. Timmusk, personal communication).

*Aspergillus niger* populations were estimated as colony counts from the peanut plant rhizosphere macerates (Domch *et al.* 1995). Briefly, five peanut plant rhizosphere macerates were dilution plated on 2% malt agar plates. After 2 days of growth at  $25^\circ\text{C}$  the colonies were transferred to fresh agar plates, three colonies per plate and grown further for 4 days when the morphological identification was performed by microscopy.

### Greenhouse experiment

Peanut plant seeds (Giza 4 cv.) were surface-sterilized using 0.3% (v/v) sodium hypochlorite for 10–12 min and then washed four times in sterile double distilled water. The seeds were then sown on water agar for the germination test. Nonsterile greenhouse potting mix soil was inoculated with *A. niger* at  $2 \times 10^4$  CFU. Peanut seeds were soaked in the *P. polymyxa* suspension for 30 min at a rate of  $10^5$  cells  $\text{ml}^{-1}$  and sown directly 1 cm below the soil surface. Peanut plants were grown for 60 days in a greenhouse at  $25^\circ\text{C}$ . Ten replicates for the inoculated and control treatments were conducted. The soil water content of each pot was adjusted daily with water, which was added onto the soil surface. The plant stand and crown rot incidence were calculated at 1 and 2 months after sowing, respectively. *Paenibacillus polymyxa* and *A. niger* population sizes were estimated in the peanut plant rhizosphere as described above.

### Microtitre plate assay for biofilm formation

Bacteria grown overnight on NA plates were resuspended in NB medium and diluted to final optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.002. Cultures were transferred to standing culture vessels. Polystyrene 96-well microtitre plates were filled with 150  $\mu\text{l}$  of culture per well. The cultures were allowed to stand at  $30^\circ\text{C}$  for the specified times. The extent of biofilm formation was assayed by staining with crystal violet. After the incubation period, cultures were removed, and microtitre plate wells were gently washed three times with 150  $\mu\text{l}$  of sterile water to remove loosely associated bacteria, then dried at  $30^\circ\text{C}$  for 30 min. Samples were stained by the addition of the 1% crystal violet solution to each well above the initial inoculation level and incubated for 20 min. The vessels were then washed. The intensity of crystal violet staining was measured after the addition of dimethyl sulfoxide to each dry well. The samples were incubated for 20 min, after which the  $\text{OD}_{590}$  values were measured on a plate reader. All samples were tested in seven independent wells.

### In vitro antagonism test

Antagonistic activity of the bacterial isolates was measured as the zone of inhibition on PDA, NA and malt extract agar (MA) with and without incorporation of 10% NaCl. The fungus was inoculated in the centre of the dish and the bacterium was inoculated near the periphery. The inhibition zones were measured after the plates were incubated for 4 days at 25, 30 and 37°C.

### A. thaliana gnotobiotic experiment; plant growth conditions and inoculation with P. polymyxa

Seeds of *A. thaliana* ecotype C24 were surface-sterilized by incubation in saturated and filtered aqueous calcium chlorate solution for 30 min, followed by repeated washes in sterile distilled water. The seeds were then sown on MS-2 medium (Murashige and Skoog 1962) for germination. Plants were then transferred to new culture dishes with MS media and grown for 2 weeks in a growth chamber at 22°C with a 16 h light regime. The light intensity was 200  $\mu\text{mol E m}^{-2} \text{s}^{-1}$ . Care was taken to maintain sterility during growth and handling of the plants.

*Paenibacillus polymyxa* strains B5 and B6 were grown in nutrient broth medium at 30°C in 100 ml flasks to late log phase. After 2 weeks of growth, plants were inoculated by soaking their roots in 10 ml diluted overnight cultures of *P. polymyxa* ( $\approx 10^5$  bacteria  $\text{ml}^{-1}$ ) and after 24 h prepared for SEM analysis (Timmusk and Wagner 1999).

### Scanning electron microscope studies

One month after seed sowing, roots and soil were collected and fixed in glutaraldehyde according to Smyth *et al.* (1990), or in a solution containing formaldehyde (Drews *et al.* 1991). The samples were dehydrated through a graded ethanol series and critical point-dried in  $\text{CO}_2$ . Pressure was decreased very slowly to prevent tissue damage. Leaf samples were mounted on stubs and shadowed with gold (22 nm) before viewing with a Philips Autoscan SEM (Tokyo, Japan). All images were computer-processed.

### Statistical analysis

The collected data were evaluated statistically using the software spss for Windows (release 7.5.1, 20 December 1996; SPSS Inc., Chicago, IL). Data were subjected to analyses of variance and treatment mean values were compared by a modified Duncan's multiple test ( $P < 0.05$ ).

Standard deviation of the mean of *A. niger* and *P. polymyxa* population sizes and inhibition zones were also determined.

## Results

### Isolation and identification of the biocontrol strain

A large number of peanut rhizosphere isolates were screened for inhibition zones on NA. A clear inhibition zone  $>3$  mm was recorded as positive. Two isolates with the inhibition zones  $>12$  mm were selected for further study. Morphologically, the isolates were rod shaped, 0.6 by 4.0  $\mu\text{m}$  Gram-positive motile bacteria which produced endospores upon prolonged incubation on agar medium. Both isolates were positive for gelatine liquefaction starch hydrolysis, glucose fermentation and catalase nitrate reduction, but negative for oxidase, urease, indole production and  $\text{H}_2\text{S}$  formation. The sequences of the two isolates were identical to each other and comparison with known bacterial sequences in NCBI GenBank using BLAST showed that the 16S rRNA gene of the isolates differed by only one nucleotide from a *P. polymyxa* strain (the difference was a change from T to C at position 483, GenBank accession number: AF515611). Thus, it was concluded that the isolates belong to *P. polymyxa* and they were given the strain designation B5 and B6.

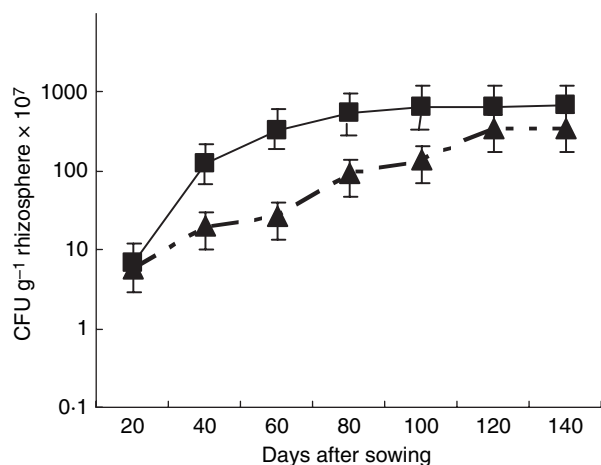
### In situ suppression of crown rot disease

Treatments with the isolates significantly reduced the disease severity compared to the pathogen control (Table 1 and Fig. 2). Both isolates were efficient in reducing *A. niger* disease intensity but B5 was more effective. Whereas

	2005			2006		
	Crown rot incidence (%)	Plant dry weight (g)	Plant yield (g)	Crown rot incidence (%)	Plant dry weight (g)	Plant yield (g)
Control	27.6	16.7	47.8	29.7	15.8	46.8
<i>P. polymyxa</i> B5	0.26	33.6	86.6	0.7	32.5	82.8
<i>P. polymyxa</i> B6	2.46	29.6	80.5	2.96	29.5	86.8
LSD	0.54	3.05	5.87	0.96	2.85	3.67

**Table 1** *Paenibacillus polymyxa* inhibition of peanut crown rot disease (field experiments)

The data represent mean values of five independent measurements. LSD, least significant difference at  $P < 0.05$ .



**Figure 1** *Paenibacillus polymyxa* colonization on peanut roots (field experiment). Two year average sizes of *P. polymyxa* B5 (■) and B6 (▲) populations in peanut plant rhizosphere. Roots were prepared and analysed as described in Materials and Methods.

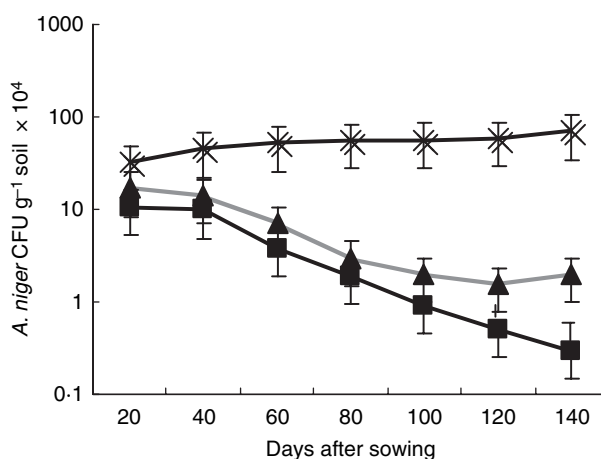
with isolate B5 the disease index reached 0.26 and 0.6, the application of B6 resulted in 2.46 and 2.96 during the 2005 and 2006 seasons, respectively.

Treatments with both isolates promoted plant growth estimated as plant yield and plant dry weight (Table 1). Generally, the plant growth parameters were higher in plants treated with *P. polymyxa* than in untreated controls during the 2005 and 2006 seasons (Table 1). The ability of *P. polymyxa* to colonize peanut rhizosphere was evaluated over 140 days. B5 isolate showed significantly higher colonization efficiency during the first 60 days than the B6 isolate (Fig. 1). After 60 days *P. polymyxa* B5 reached a density of  $5 \times 10^9$  cells g<sup>-1</sup> of rhizosphere soil while the density of *P. polymyxa* B6 isolate slowly increased during the growing season, reaching  $10^9$  cells g<sup>-1</sup> soil only at the end of the season after 120 days (Fig. 1).

The colonization by *A. niger* in the presence or absence of each of the two isolates of *P. polymyxa* was assessed throughout the 140-day growth period (Fig. 2). The *A. niger* counts reached  $10^7$  in the rhizosphere of untreated plants after 140 days from sowing (Fig. 2). *Aspergillus niger* density was reduced to  $3 \times 10^3$  in the rhizosphere treated with the cells of *P. polymyxa* B5 after 140 days of sowing. At the same time of counting (140 days from sowing) *P. polymyxa* B6 had also caused remarkable fungus reduction with *A. niger* counts of  $3 \times 10^4$  in the peanut rhizosphere.

### Greenhouse experiment

The colonization by *A. niger* of the peanut rhizosphere in the presence or absence of each of the two isolates of



**Figure 2** *Aspergillus niger* inhibition by *Paenibacillus polymyxa* (field experiment). Two year average sizes of *A. niger* populations in peanut plant rhizosphere under field conditions. Roots were prepared and analysed as described in Materials and Methods. ■, *P. polymyxa* (B5); ▲, *P. polymyxa* (B6); ×, untreated control.

*P. polymyxa* was assessed throughout the 60-day growth period (Table 2). Crown rot disease incidence was dramatically decreased when the seeds were soaked with *P. polymyxa* strains with the better results being obtained with strain B5 (Table 2). The results correlate with the *A. niger* counts, which were  $2 \times 10^6$  cells g<sup>-1</sup> rhizosphere of untreated plants after 30 days and  $7 \times 10^8$  after 60 days from sowing (Table 2). After 30 days from sowing, *A. niger* was nondetectable in rhizosphere treated with *P. polymyxa* B5 and showed a count of  $10^2$  in rhizosphere treated with strain B6 (Table 2).

The ability of *P. polymyxa* to colonize peanut roots in greenhouse was also measured and the results reveal a similar pattern as in the field experiment. The strain B5 showed significantly better colonization ability and  $10^9$  bacteria per gram soil was reached after 30 days. The *P. polymyxa* B6 density also increased gradually in the rhizosphere of peanut plant, reaching a density of  $10^9$  cells g<sup>-1</sup> rhizosphere at 60 days from sowing (Table 2). The population sizes required to antagonize *A. niger* were estimated. It was found that  $10^5$  *P. polymyxa* cells per gram rhizosphere efficiently antagonized *A. niger*. The inoculum density for strain B6 was  $10^7$ .

### In vitro antagonism test

The antagonistic activity of the bacterial isolates was measured as the zone of inhibition on PDA, NA and MA with and without NaCl addition. The colony diameters were determined after 4 days of growth at 25, 30 and 37°C. The *A. niger* inhibition zone varied from 13 to 20 mm depending on the medium used and incubation

**Table 2** *Paenibacillus polymyxa* inhibition of peanut crown rot disease (greenhouse experiment)

	Plant stand (%)*	Crown rot incidence (%)	<i>Aspergillus niger</i> CFU g <sup>-1</sup> peanut rhizosphere		<i>P. polymyxa</i> CFU g <sup>-1</sup> peanut rhizosphere	
			30 days	60 days	30 days	60 days
Pathogen control	47.9	28.6	$2 \times 10^6 \pm 0.4 \times 10^6$	$7 \times 10^8 \pm 0.3 \times 10^8$		
<i>P. polymyxa</i> B5	99.4	ND			$2 \times 10^9 \pm 0.4 \times 10^9$	$3 \times 10^9 \pm 0.5 \times 10^9$
<i>P. polymyxa</i> B6	99.5	ND			$3 \times 10^7 \pm 0.3 \times 10^7$	$2 \times 10^9 \pm 0.4 \times 10^9$
<i>P. polymyxa</i> B5 + <i>A. niger</i>	99.5	0.25	ND	ND	$3 \times 10^9 \pm 0.4 \times 10^9$	$5 \times 10^9 \pm 0.6 \times 10^9$
<i>P. polymyxa</i> B6 + <i>A. niger</i>	98.6	1.06	$2 \times 10^2 \pm 0.3 \times 10^2$	$2 \times 10^2 \pm 0.4 \times 10^2$	$2 \times 10^7 \pm 0.3 \times 10^7$	$2 \times 10^9 \pm 0.4 \times 10^9$

The data represent mean values of five independent measurements.

\*Plant stand was estimated 1 month after sowing. Crown rot incidence was estimated 2 months after sowing.

ND, not detectable.

temperature but was relatively similar for both *P. polymyxa* isolates (data not shown).

#### Microtitre plate assay for biofilm formation

An assay for biofilm formation on solid surfaces was performed on microtitre plates. Previous studies had indicated that the crystal violet assay could be used to measure the initiation of biofilm formation and biofilm maturation (Friedman and Kolter 2004). Figure 4 shows a time course of biofilm formation as assayed by crystal violet staining and gentle washing of standing cultures grown at 30°C. During the first 10 h, the B5 strain of *P. polymyxa* initiated biofilm formation in a manner similar to that of the B6 strain. After the period the B6 strain started to show a significant decrease in staining, suggesting a remarkably lower ability to form biofilms than strain B5. In contrast, strain B5 biofilm enlarged continuously throughout the experimental period.

#### *A. thaliana* gnotobiotic experiment

The results for the counts of viable *P. polymyxa* B5 and B6 in the peanut rhizosphere during two seasons (Fig. 1) indicate the higher rhizosphere colonization rate of isolate B5 in the experiments. When we compared the ability to form biofilms of the two isolates on solid surfaces we found that isolate B5 was also more efficient at forming extracellular matrices (Fig. 4). Plant roots are not passive targets for bacteria. It has been shown earlier that *P. polymyxa* colonization and biofilm formation on *A. thaliana* gnotobiotic model system faithfully reflects the bacterial colonization in soil (Timmusk and Wagner 1999). Hence, in addition to abiotic solid surfaces we studied the ability of the two isolates to form biofilms on *A. thaliana* roots in our model system. The SEM results (Fig. 3g,h) confirm the results on solid surfaces (Fig. 4) showing that the *P. polymyxa* B5 isolate is an effective biofilm former.

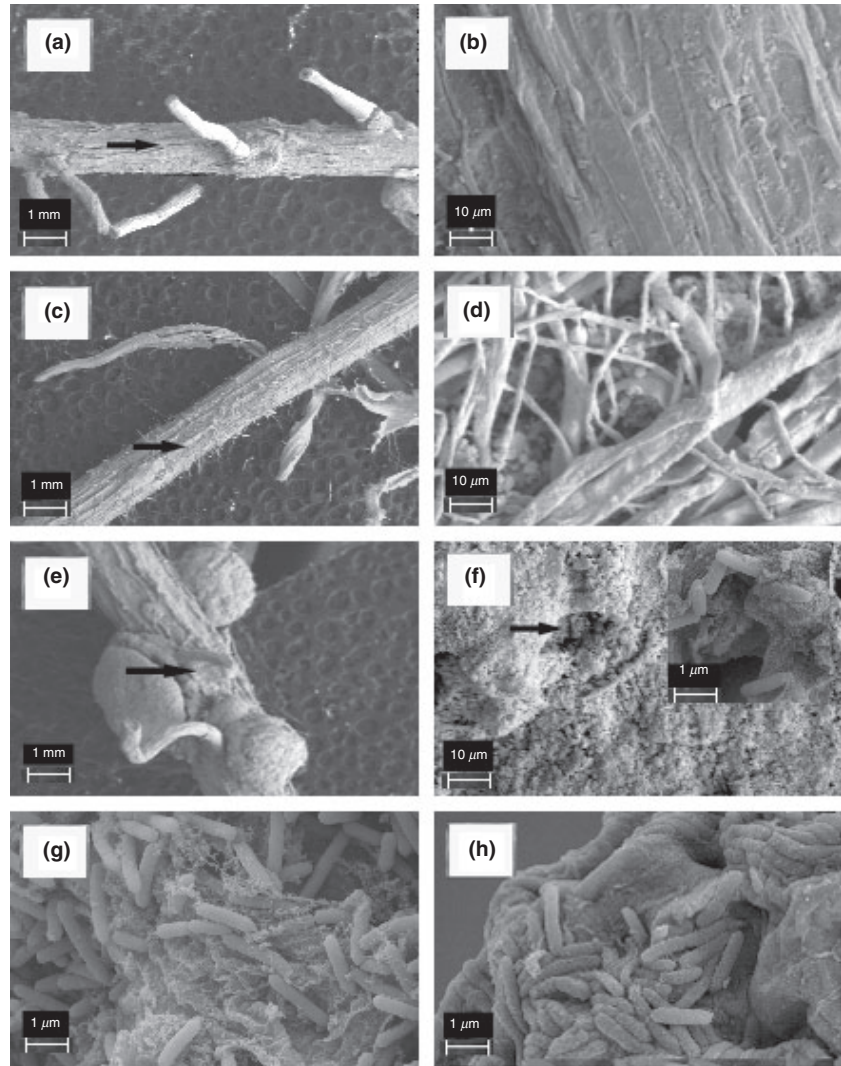
#### Discussion

Recently, it has often been stressed that the underground resources of the plant rhizosphere could offer new opportunities for biotechnology in agriculture (Deutschbauer *et al.* 2006; Stahl and Wagner 2006). Numerous studies have described the isolation of micro-organisms and demonstrated their potential to antagonize *A. niger* under laboratory conditions. However, only a few of these reports have demonstrated field efficiency and an even smaller subset have given rise to commercial applications. It is often suspected that biocontrol agents do not perform well under field conditions (Brunner *et al.* 2005).

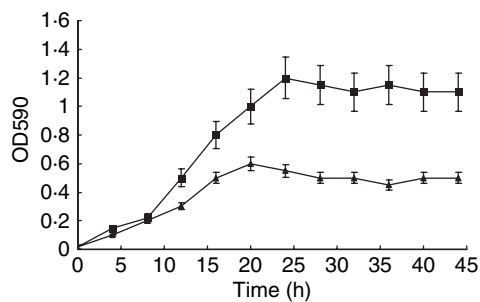
Here, we report on the ability of two *P. polymyxa* strains to antagonize crown root rot caused by *A. niger* under field conditions during two growth seasons. We have shown that peanut plant growth is promoted and the incidence of the pathogen is reduced (Tables 1 and 2 and Fig. 2) in rhizosphere inoculated with *P. polymyxa* strain B5 or B6.

The two *P. polymyxa*, morphologically and biochemically similar strains, differed in their ability to antagonize *A. niger* in soil systems (Tables 1 and 2 and Fig. 2). There are many reports on the antagonistic abilities of *P. polymyxa* against various pathogens. Mostly this ability is linked to the bacterial antibiotic production. It is well known that the antibiotic compounds produced by *P. polymyxa* are able to out-compete both Gram-positive and Gram-negative microbiota (Timmusk and Wagner 1999; He *et al.* 2007). Hence, we studied the ability of these strains to inhibit *A. niger* on agar plates and HPLC (data not shown). As indicated by the inhibition zones and by the HPLC chromatograms, antibiotic compounds are produced in similar quantities by both strains.

To make biocontrol effective and reproducible, successful colonization with the biocontrol agent must be ensured. Hence, we used SEM and solid surface assay to study colonization and biofilm formation ability of the



**Figure 3** Scanning electron micrographs of plant roots colonized by *Paenibacillus polymyxa* and *Aspergillus niger* (greenhouse experiment and gnotobiotic experiment). Peanut plant roots (a and b) in potting soil were infected with *A. niger* (c and d). *Paenibacillus polymyxa* B5 biofilm formation on peanut plant inhibits *A. niger* colonization (e and f). *Paenibacillus polymyxa* B5 (g) and B6 (h) biofilm formation on *A. thaliana* root. Roots were prepared and visualized as described in Materials and Methods. Note that *P. polymyxa* B5 isolate produces remarkably more extracellular matrix. Arrows indicate further magnification sites.



**Figure 4** Solid surface assay of *Paenibacillus polymyxa* B5 (■) and B6 (▲) biofilm formation. The crystal violet assay was used to measure the solid surface biofilm formation at 30°C. Preparation and analysis as described in Materials and Methods.

strains (Figs 3 and 4). The strain B5 produces strikingly larger amounts of extracellular polymers (Fig. 4). Earlier we have shown that *P. polymyxa* colonizes plant roots as

biofilm (Timmusk and Wagner 1999). We have studied the pattern and suggested its role in biocontrol (Timmusk and Wagner 1999; Timmusk *et al.* 2005). The data here support our former results. It is of course clear that results on biofilm quantification on solid surface assays cannot be automatically transposed to soil conditions as the conditions differ. However, the SEM studies from biological settings (Fig. 3) also show that strain B5 is the better biofilm former.

In this study, both strains were isolated from the peanut rhizosphere. Yet they differed in their ability to colonize (Table 2 and Fig. 1). Likewise as expected they also differed in their ability to colonize on the *A. thaliana* model system which has been shown to faithfully reflect natural conditions (Timmusk and Wagner 1999; S. Timmusk, unpublished data). This might suggest that the greater ability of strain B5 to antagonize *A. niger* reflects its higher population densities in the rhizosphere. But at

population densities of  $10^5$ , B5 antagonized *A. niger* (data not shown), whereas B6 did not, even though antibiotics were produced at similar rates (manuscript in preparation). These results, together with those from SEM, support the idea that niche exclusion is involved in the biocontrol (Fig. 1).

A biocontrol bacterium can affect plant growth by various mechanisms (Glick *et al.* 1999). Most likely the plant uses different abilities for growth promotion at various times (Glick *et al.* 2001). This makes the task of finding the mechanism under given conditions of environment, plant and pathogen and biocontrol agent particularly challenging. All these players are dynamic, and real-time monitoring over the growth period of various parameters, including, e.g. antibiotic production, would be needed to estimate the role of different factors. *Paenibacillus polymyxa* produced antibiotic compounds certainly contribute to its antagonism against various pathogens in different natural systems. If nothing else they certainly facilitate colonization through biofilm formation, resulting in the protection of pathogen infection sites (Timmusk and Wagner 1999; Timmusk 2003; Timmusk *et al.* 2005). However, the results indicate the possibility that these compounds might not always be the primary cause of biocontrol.

Competition for colonization sites and nutrients supplied by root exudates is probably a significant factor in most interactions between plant beneficial bacteria and pathogens. Yet this mechanism is often overlooked because it is difficult to study in biological systems. How the antibiotic production and biofilm formation of *P. polymyxa* are related to biocontrol ability needs careful testing. We shall continue FTIR and NMR studies to evaluate compounds produced by the strains in natural system. It is hoped that field experiments employing B5 biofilm-deficient mutants will bring more light to the issue. We are currently identifying the genes involved in *P. polymyxa* biofilm formation from Tn10 mutagenesis experiments.

In conclusion, the field and greenhouse experiments in agreement with the laboratory studies demonstrated remarkable protection against crown root rot. As both isolates are nonpathogenic growth-promoting natural isolates, they may prove ideal for fighting damaging crown root disease.

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## References

- Alvarez, V.M., von der Weid, I., Seldin, L. and Santos, A.L. (2006) Influence of growth conditions on the production of extracellular proteolytic enzymes in *Paenibacillus peoriae* NRRL BD-62 and *Paenibacillus polymyxa* SCE2. *Lett Appl Microbiol* **43**, 625–630.
- Ash, C., Priest, F.G. and Collins, M.D. (1993) Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie Van Leeuw* **64**, 253–260.
- Branda, S.S., Vik, S., Friedman, L. and Kolter, R. (2005) Biofilms: the matrix revisited. *Trends Microbiol* **13**, 20–26.
- Brunner, K., Zeilinger, S., Ciliento, R., Woo, S.L., Lorito, M., Kubicek, C.P. and Mach, R.L. (2005) Improvement of the fungal biocontrol agent *Trichoderma atroviride* to enhance both antagonism and induction of plant systemic disease resistance. *Appl Environ Microbiol* **71**, 3959–3965.
- Carina, M., Andrea, A., Lorena, P., Maria, G., Stella, C. and Ana, C. (2006) Ochratoxin A and the occurrence of ochratoxin A producing black aspergilli in stored peanut seeds from Cordoba, Argentina. *J Agron Crop Sci* **86**, 2369–2373.
- Deutschbauer, A.M., Chivian, D. and Arkin, A.P. (2006) Genomics for environmental microbiology. *Curr Opin Biotechnol* **17**, 229–235.
- Domch, K.H., Gams, W. and Anderson, T.H. (1995) *Compendium of Soil Fungi*. Port Jervis, NY: Lubrecht & Cramer Ltd.
- Drews, G.N., Bowman, J.L. and Meyerowitz, E.M. (1991) Negative regulation of the *Arabidopsis* homeotic gene AGAMOUS by the APETALA2 product. *Cell* **65**, 991–1002.
- Elwakil, M.A. (2003) Use of antioxidant hydroquinone in the control of peanut seed-borne fungi. *Pak J Plant Pathol* **2**, 75–79.
- Emmert, E.A.B. and Handelsman, J. (1999) Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiol Lett* **171**, 1–9.
- Friedman, L. and Kolter, R. (2004) Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* **51**, 675–690.
- Gamalero, E., Lingua, G., Berta, G. and Lemanceau, P. (2003) Methods for studying root colonization by introduced beneficial bacteria. *Agronomie* **23**, 407–418.
- Gau, A.E., Dietrich, C. and Kloppstech, K. (2002) Non-invasive determination of plant-associated bacteria in the phyllosphere of plants. *Environ Microbiol* **4**, 744–752.
- Glick, B.R., Patten, C.L., Holguin, G. and Penrose, D.M. (1999) *Biochemical and Genetic Mechanisms used by Plant Growth-promoting Bacteria*. London: Imperial College Press.

- Glick, B.R., Penrose, D.M. and Ma, W. (2001) Bacterial promotion of plant growth. *Biotechnol Adv* **19**, 135–138.
- Haggag, W. and AboSedera, S.A. (2000) Influence of iron sources and siderophores producing *Pseudomonas fluorescens* on crown rot. *Egypt J Microbiol* **28**, 1–16.
- He, Z., Kisla, D., Zhang, L., Yuan, C., Green-Church, K.B. and Yousef, A.E. (2007) Isolation and identification of a *Paenibacillus polymyxa* strain that coproduces a novel antibiotic and polymyxin. *Appl Environ Microbiol* **73**, 168–178.
- Kolter, R. and Greenberg, E.P. (2006) Microbial sciences: the superficial life of microbes. *Nature* **441**, 300–302.
- Lindberg, T. and Granhall, U. (1984) Isolation and characterization of dinitrogen-fixing bacteria from the rhizosphere of temperate cereals and forage grasses. *Appl Environ Microbiol* **48**, 683–689.
- Lucy, M., Reed, E. and Glick, B.R. (2004) Applications of free living plant growth-promoting rhizobacteria. *Antonie Van Leeuwenhoek* **86**, 1–25.
- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* **15**, 473–497.
- O'Toole, G.A., Pratt, L.A., Watnick, P.I., Newman, D.K., Weaver, V.B. and Kolter, R. (1999) Genetic approaches to study of biofilms. *Methods Enzymol* **310**, 91–109.
- O'Toole, G., Kaplan, H.B. and Kolter, R. (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* **54**, 49–79.
- Smyth, D.R., Bowman, J.L. and Meyerowitz, E.M. (1990) Early flower development in *Arabidopsis*. *Plant Cell* **2**, 755–767.
- Stahl, D.A. and Wagner, M. (2006) The knowledge explosion in environmental microbiology offers new opportunities in biotechnology. *Curr Opin Biotechnol* **17**, 227–228.
- Sugita, C., Makimura, K., Uchida, K., Yamaguchi, H. and Nagai, A. (2004) PCR identification system for the genus *Aspergillus* and three major pathogenic species: *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. *Med Mycol* **42**, 433–437.
- Timmusk, S. (2003) *Mechanism of action of the plant growth promoting bacterium Paenibacillus polymyxa*. PhD Thesis. Uppsala University, Uppsala.
- Timmusk, S. and Wagner, E.G. (1999) The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. *Mol Plant Microbe Interact* **12**, 951–959.
- Timmusk, S., Nicander, B., Granhall, U. and Tillberg, E. (1999) Cytokinin production by *Paenibacillus polymyxa*. *Soil Biol Biochem* **31**, 1847–1852.
- Timmusk, S., van West, P., Gow, N.A.R. and Wagner, E.G.H. (2003) Antagonistic effects of *Paenibacillus polymyxa* towards the oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*. In *Mechanism of Action of the Plant Growth Promoting Bacterium Paenibacillus polymyxa*. PhD thesis. Uppsala: Uppsala University.
- Timmusk, S., Grantcharova, N. and Wagner, E.G.H. (2005) *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Appl Environ Microbiol* **11**, 7292–7300.
- Weisburg, W.G., Barns, S.M., Pelletier, A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 679–703.
- Weller, D.M. and Thomashow, L.S. (1994) Current challenges in introducing beneficial microorganisms into the rhizosphere. In *Molecular Ecology of Rhizosphere Microorganisms* ed. O'Gara, F., Dowling, D.N. and Boesten, B. pp. 1–18. NY: Academic Press.
- Yuen, K.Y., Godoy, G., Steadman, J.R., Kerr, E.D. and Craig, M.L. (1991) Epiphytic colonisation of dry edible bean by bacteria antagonistic to *Sclerotinia sclerotinum* and potential for biological control of white mold disease. *Biol Control* **1**, 293–301.